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(54) Title: PROCESS FOR PRODUCING ANTI-FREEZE PEPTIDES

(57) Abstract

Process for producing anti-freeze peptides (AFP), by expressing in a host organism, preferably a yeast, a gene encoding AFP controlled by a strong promoter, e.g. an inducible GAL7 or a constitutive GAPDH promoter of *Saccharomyces cerevisiae*. The AFP gene can be preceded by a signal sequence for secretion of the protein, e.g. the invertase signal sequence or the pre-sequence of the α-mating factor of *S. cerevisiae*. The non-AFP part in the fused gene can be part of the α-galactosidase gene of *Cyamopsis tetragonoloba*. The gene construct can comprise a processing site that can be used to split off at least one AFP from the remainder of the protein. The AFP gene can be present as a tadem-repeat of AFP genes. The invention also relates to AFP produced as described above and to the use of such AFP for inhibiting ice crystal growth, e.g. in ice cream and other food products, or other biological materials that can be frozen. And further to a yeast having improved freeze tolerance, containing and/or surrounded by AFP produced as described above, which can be used in producing frozen dough or other compositions that can be frozen.

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Title: Process for producing anti-freeze peptides.

INTRODUCTION

Technical Field

The invention relates to transforming host cells, in particular yeast cells, for expression and secretion of peptides which inhibit ice crystal growth.

Background and prior art

The success of a commercial process for the production of recombinant proteins requires a non-pathogenic organism amendable to large scale, high density fermentation, e.g. *Saccharomyces cerevisiae*. Ideally, while the host organism should secrete relatively few proteins into the culture supernatant that could countermined or proteolytically degrade the product, it should have the capacity to secrete large quantities of correctly processed recombinant protein into the medium.

Antifreeze proteins (AFP's) from polar fish are characterized by their property of causing a noncolligative depression of freezing point, and beside this, these molecules inhibit the recrystallization of ice (Knight *et al.*, 1984). Both types of activity are thought to result from the interaction of these cryoprotectants with the ice crystal.

Within the winter flounder two different AFP's predominate, the HPLC-6 and the HPLC-8 (Fourney *et al.*, 1984), both belonging to the type I class of AFP's (Davies *et al.*, 1990), and they are the only one for which a x-ray crystal structure is known, and for which detailed structure/function relationships have been proposed (Yang *et al.*, 1988,). In general, the 37 amino acids long molecule contains three 11 amino acids tandem repeats, with mainly alanine amino acids that favour a single α helix with a moderate amphiphilic character, having the majority of the hydrophillic amino acids along one face of the molecule, that are potential sites for hydrogen bonding to the oxygen atoms of the ice lattice.

The AFP binds preferentially to the prism face, and thereby blocks the ice growth at the preferred growth site. The created dipole moment is thought to be the initial driving force for the specific recognition. The induced ordering of the water dipoles within the ice only allows further ice growth on the unordered basal plane, where

then AFP's bind again. This growth habit alteration causes the development of bipyramidal ice crystals, which are significantly smaller than normal water crystals (Davies *et al.*, 1990).

Within *E.coli* fusions of synthetic or semisynthetic genes encoding antifreeze proteins and either truncated staphylococcal protein A gene (Mueller *et al.*, 1991; Warren *et al*, 1990) or pro AFP's with ß-galactosidase (Peters *et al.*, 1989) were expressed intracellular . Different chimeric proteins containing different numbers of the 11 amino acid imperfect repeats were linked to the C-terminal end of protein A by a short amino acid spacer and tested for their recrystallization inhibition potential, whereby a protein with only two repeats lacked activity, while the inhibitory activity increased progressively for proteins containing three, four and five repeats, indicating that the number of homologous segments may determine the effectiveness of a single molecule in suppressing crystal growth, or may determine the molar number of molecules necessary to have an effect.

Warren *et al.* also tested the expression of a slightly modified AFP-6 as an internal fusion with ß-galactosidase in *E.coli.* Crude extract of the transformed cells displayed thereby a five-fold greater recrystallization inhibition than the negative control within the splat assay.

For the foreign gene expression and secretion in yeast, the invertase (SUC2) signal sequence (Perlmann at al., 1982) has been used widely. Human α2-IFN (Chang *et al.*, 1986) and human single chain urinary plasminogen activator (Melnick *et al.*, 1990) have successfully secreted using this sequence, while the secretion of human α-1 anti-trypsin behind the SUC2 sequence caused problems (Moir *et al.*, 1987). Attachment of a heterologous protein to the signal sequence often results in a change of at least the first amino acid C-terminal to the signal peptidase cleavage side. However, there appears to be flexibility in recognition of cleavage junction, since fusions of SUC2 with altered amino acid junctions, such as HSA or two forms of insulin, are still cleaved (Hitzeman *et al.*, 1990).

The second well described option for the expression and secretion of proteins and peptides within *S. cerevisiae* is the application of the pre-pro sequence of the α mating factor, one of the two oligopeptide hormones (pheromones) triggering sexual conjugation of the haploid cell types a and α (Betz *et al.*, 1981). α-factor is a

13-amino acid peptide that is encoded by two unlinked structural genes, MFα1 and MFα2. These genes encode precursors of 165 and 120 amino acids, respectively (Caplan *et al.*, 1991). Both precursors include a signal, a pro region with three sites for N-glycosylation, and tandem α-factor repeats each of which is preceded by a spacer peptide. The 19 amino acid signal (pre) sequence is responsible for the initial targeting of the molecule to the secretory pathway, and is cut off by a signal peptidase, and after processing of the pro-part through proteolytic activities including Kex2 endoproteinase, Kex1 carboxypeptidase and the dipeptidylpeptidase aminopeptidase in the golgi, the mature α mating factor is released into the surrounding medium.

The α leader segment mediated secretion from *S. cerevisiae* is described in the literature for several different proteins and peptides, for example a consensus α-IFN, ß-endorphin (Bitter *et al.*, 1984), human-insulin like growth factor I expression (Steube *et al.*, 1991), and human interleukin-6 expression (Guisez *et al.*, 1991). The potential of α-MF leader sequence for high secretion, seems to be rather low (Rothstein *et al.*, 1987), although more recent experiments indicate the specific relationship of leader sequence and structural protein to be more crucial for the success of such a process (Sleep *et al.*, 1990). Moreover, recent results indicate the possibility that for some fusions the deletion of the complete pro-peptide sequence increases the secretion efficiency significantly (Achstetter *et al.*, 1992).

SUMMARY OF THE INVENTION

According to one embodiment of the invention, a desired gene encoding a protein active in the inhibition of ice crystal growth is placed in frame as a fusion construct behind the α-galactosidase gene of *Cyamopsis tetragonoloba*. In order to be capable to regulate expression the new chimeric gene is placed under the control of the strong inducible GAL7 promoter. To obtain secretion of the α-galactosidase-AFP fusion protein into the surrounding medium the whole gene is placed behind the invertase signal sequence.

According to another embodiment of the invention a gene encoding the desired anti-freeze peptide is directly expressed under the control of the inducible GAL7

promoter. To receive secretion the gene coding for the mature AFP is separated by the pre-pro sequence of the α mating factor from the promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. a) Schematic presentation of plasmid pUR2650.
b) schematic presentation of plasmid pUR2651.
Abbreviations used: a-gal: synthetic gene coding for α-galactosidase from *Cyamopsis tetragonoloba*. amp r.: ß-lactamase gene, arrows indicate the direction of transcription, the size of the plasmid is mentioned in the Figure; the small box in front of the α-galactosidase gene represents the SUC2 signal sequence. afp: DNA coding for one copy of a synthetic AFP gene, the thicker line between α-galactosidase gene and AFP gene represents the IEGR sequence.

Figure 2. a) Schematic presentation of plasmid pUR2652, Xa-AFP indicates here the IEGR AFP coding sequence; LEU2d indicates the LEU2 gene with the truncated promoter. b) Schematic presentation of plasmid pUR2653.
Abbreviations used: GAL7-ISS: Gal7 promoter and SUC2 signal sequence; 2um = 2u = 2μm, the DNA coding for AFP is indicated as a solid box. LEU2 indicates the LEU2 gene with the intact promoter. T-pgk = T-PGK represents the PGK terminating sequence.

Figure 3. a) Schematic presentation of plasmids pUR2660 containing 2 copies of AFP separated by KREA. b) plasmid pUR2665, containing 3 copies of AFP with the internal KREA spacers.

Figure 4. a) Schematic presentation of plasmid pUR2666, containing 4 copies of AFP and the internal KREA spacers and b) schematic presentation of plasmid pUR2667, containing 5 copies.
Abbreviations used are the same than in the description of Figure 1 and Figure 2.

Figure 5. Schematic presentation of plasmid pUR2674 containing the fusion between α-galactosidase and 5 copies of AFP, separated by the last 7 amino acids of the α-mating factor pro-sequence.

Figure 6. a) Schematic presentation of plasmids pUR2676.
b) pUR2677, the plasmids containing either 2 or 4 directly linked AFP gene cassettes behind the GAL7 promoter and the SUC2 signal sequence.

Figure 7. a) Schematic presentation of plasmids pUR2678.
b) pUR2679; the plasmids containing either 2 or 4 directly linked AFP gene cassettes behind the GAL7 promoter and the pre-pro α-mating factor sequence.

Figure 8. a) SDS gel analysis. b) Western analysis of soluble cell fractions and culture fluids of yeast cells containing pUR2652.
Separation on a 8-18% PAA gradient gel. α-gal: purified α-galactosidase; c: soluble cell fraction; s: supernatant of cell cultures. b) The immune specific stain was done with anti-AFP antibody. The arrow indicates the band of the fusion construct.

Figure 9. a) SDS gel analysis. b) Western analysis of soluble cell fractions of 3 different transformants and 1 culture fluids of yeast cells containing pUR2653.
Separation on a 8-18% PAA gradient gel. α-gal: purified α-galactosidase; c: control= soluble cell fraction of host strain *S. cerevisiae* SU10; t: 3 different soluble cell fractions of transformants containing pUR2653. s: supernatant of cell culture.of transformant 1, 2 and 3 not shown, because identical. b) The immune specific stain was done with anti-AFP antibody. c: control= culture fluid of untransformed SU10 cultures. The arrow indicates the band of AFP.

Figure 10. Graphical presentation of the results of the thermal hysteresis assay. The difference in °C between freeze- and melting point is given on the vertical axis.
A: chemically synthesized purified AFP at a final concentration of 3%.
B: purified α-galactosidase at a final concentration of 3%.
C: An about 40% suspension of freeze dried culture fluid of yeast cultures containing pUR2652.
D: an about 40% suspension of freeze dried culture fluid of yeast cultures containing pUR2653. For further details see text.

Figure 11. Recrystallization assay of culture fluid from yeast cells containing pUR2652.
A: 30% sucrose, 1). before and 2) after incubation.
B: 3% chemically synthesized AFP in 30% sucrose 1) before and 2) after incubation.
C: about 40% freeze dried culture medium from cells harbouring pUR2652, a) before and b) after incubation. magnification were identical in all cases.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The invention particularly relates to a process for producing peptides inhibiting ice crystal growth, so-called anti-freeze peptides (AFP), by expressing in a host organism a gene encoding an AFP or encoding a fusion protein comprising an AFP, which comprises

(i) forming a DNA construct comprising in the order given

(a) a strong, optionally inducible, promoter active in the host organism,

(b) an ATG start codon, that may be contained in an optionally present DNA signal sequence capable of secreting the protein produced by the host organism during expression of an AFP-encoding gene of (c) below, which signal sequence can be homologous or heterologous to the AFP-encoding gene and is in reading frame with the ATG start codon,

(c) at least one desired AFP-encoding gene in reading frame with the ATG start codon, and

(d) a stop codon bound to the 3' end of the AFP-encoding gene,

(ii) transforming a host organism with the DNA construct of (i) in such a way that the host organism can express at least the AFP-encoding gene,

(iii) growing the transformed host organism under conditions whereby the expression of at least the AFP-encoding gene occurs or is induced, and

(iv) optionally collecting the produced AFP or fusion protein, or peptide or protein obtained therefrom by further processing.

The "host organism" can be a plant or animal cell or a microorganism. Preferably a yeast, e.g. *S. cerevisiae* is used as the host organism. Transformation systems for these host organisms are well known to a skilled person and are described in the prior art (see above).

In case of a "fusion protein" good results were obtained with a process according to the invention, in which the DNA construct contains a fused gene comprising at least part of the α-galactosidase gene of *Cyamopsis tetragonoloba* and at least one AFP-encoding gene with its 5' end in-frame-fused to the 3' end of the at least part of the α-galactosidase gene. The fused gene was preceded by the invertase signal sequence of *S. cerevisiae*.

The "promoter" can be an inducible GAL7 promoter or a constitutive GAPDH promoter, both of *S. cerevisiae*. The advantage of an inducible promoter is that first sufficient biomass can be formed of the host organism which can then be induced to express the desired gene. However, a constitutive promoter is preferable under conditions where it is advantageous to express the desired gene in all growth stages. Of course the gene should comprise or be preceded by an "ATG start codon" for starting proper translation of the mRNA.

If the gene or fused gene is to be secreted a DNA "signal sequence" can be used. Most signal sequences precede the structural gene and start with an ATG start codon. As DNA signal sequence can be used for example the invertase signal sequence of *S. cerevisiae* or the DNA pre-sequence of the α-mating factor of *S. cerevisiae*. In the latter case the DNA construct can also comprise the pro-sequence of the α-mating factor of *S. cerevisiae* between the pre-sequence and the AFP-encoding gene, whereby the pre-sequence, the pro-sequence and the AFP-encoding gene are in the same reading frame.

When it is desirable that the AFP remains in the cell of the host organism and secretion is less desirable, a signal sequence need not be present. Of course the AFP need then not be collected from the host organism. On the other hand, when the presence of the AFP is desirable, a signal sequence is preferred for obtaining efficient secretion. Thus the presence of a signal sequence is optional depending on the purpose of the AFP production. Any signal sequence present can be both homologous to the AFP, thus corresponding to the AFP in its natural occurrence, or can be heterologous to the AFP. The latter can have the advantage that it is better recognized by the host organism.

For proper processing after secretion it can be advantageous when the connection between two or more genes comprises a DNA sequence encoding a "processing site" that can be used to split off at least one AFP from the remainder of the protein, either during or after secretion of the fusion protein or another precursor-AFP. It should be noted that in this specification the "at least one AFP-encoding gene" can comprise a tandem-repeat of one or more AFP-encoding genes, whereby optionally one or more AFP-encoding genes can be separated by a DNA sequence encoding a processing site.

The invention also relates to an AFP prepared by a process according to the invention and to the use of such AFP for inhibiting ice crystal growth, e.g. in ice cream and other food products, or other biological materials that can be frozen.

Another embodiment of the invention relates to a yeast having improved freeze tolerance, containing and/or surrounded by AFP produced according to the invention, which can be used in producing frozen dough or other compositions that can be frozen.

The invention is illustrated by the following examples without being limited thereto.

Mostly standard methods were used as described in Sambrook, J., Maniatis, T., Fritsch, E.F.; Molecular Cloning; Second edition; Cold Spring Harbour Laboratory Publ. (1989). If modifications were used, then they are described below.

Strains, plasmids and growth conditions

E. coli strain JM109 (endA1, recA1, syrA96, thi, hsdR17, rk^-, mk^+ relA1 supE44, Yanish-Perron *et al.*, 1985) was used for amplification of plasmids. Transformation of *JM109* was according to Chung *et al.*, 1989. *S. cerevisiae* strain SU10 (matα, cir^+, MEL^+, leu2, his3, ura3) was transformed with the plasmids pUR2652 and pUR2653. *S. cerevisiae* SU50 (mata, cir^o, leu2, his4, can1; Erhart and Hollenberg, 1981) was used for transformation of the multicopy integration plasmid pUR6803.

Transformation of the yeast strains was performed by the spheroplast method of Beggs *et al.* or by electroporation, mainly as described by Becker *et al.*, 1991. Transformants were recovered on selective YNB-plates (0.67% YNB, 2% glucose, 2% agar) supplemented with the essential amino acids (histidine 20µg/ml, uracil 20µg/ml). The same liquid medium was used for pre-cultures, 2 times overnight at 30°C and diluted 1:10 in YP medium containing 5% galactose for induction of the GAL7 promoter. Restriction enzymes and DNA modification enzymes were applied as recommended by the supplier.

Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and purified by standard procedures.

EXAMPLE 1: Construction of pUR2652

Plasmid pUR2652 was constructed to obtain a C-terminal fusion of AFP to α-galactosidase, a protein from which is known, that it is efficiently secreted into the medium, when fused to the invertase signal sequence (Verbakel, 1991). The invertase signal sequence directs proteins to the secretory pathway, and will be cut of by a signal peptidase. On the DNA level the whole gene is controlled by the inducible GAL7 promoter. For the expression of AFP-6 in *S. cerevisiae* a set of 8 oligonucleotides was synthesized (AFL 03, AFP 01, AFP 03, AFP 05, AFL 04, AFP 02, AFP 04, AFP 06), mainly comprising the DNA sequence of the mature AFP expressed in preferentially used *S. cerevisiae* codons (see SEQ.ID. No. 1 and 2).

```
     ┌ AFL03                                        ┬  AFP01
StyI CAAGGTCT ATT GAA GGT AGA GAT ACT GCT TCT GAT GCC GCC GCT GCC GCT
       CAGA TAA CTT CCA TCT CTA TGA CGA AGA CTA CGG CGG CGA CGG CGA
         └      AFL04                                              ┴

                      ┬                                AFP03      ┐┌
GCT TTG ACA GCT GCT AAT GCT AAA GCC GCT GCT GAA TTG ACA GCT GCC AAT
CGA AAC TGT CGA CGA TTA CGA TTT CGG CGA CGA CTT AAC TGT CGA CGC TTA
            AFP02                  ┴            AFP04
                           AFP05
GCA GCC GCT GCT GCT GCA GCT ACT GCG AGA TAATA
CGT CGG CGA CGA CGA CGT CGA TGA CGC TCT ATTATTCGA  HindIII
    ┘└             AFP06
```

The cassette further contained at the 5' end codons for the in frame expression of the amino acid sequence IEGR, which is known to be the recognition and cleavage sequence for the blood factor Xa (Nagai *et al.*, 1987). This construction allowed the later release the pure AFP from the fusion protein. For cloning purposes the nucleotides for the C-terminus of α-galactosidase from the restriction site *Sty*I were also included. The 3' end of the cassette included 2 stop codons, which partially overlap the *Hin*dIII restriction site. To facilitate the ligation of the assembled cassette behind the α-galactosidase gene, the *Sac*I/*Hin*dIII fragment of pUR2740, comprising the 3' end of the GAL7 promoter, the SUC2 signal sequence and the complete synthetic α-galactosidase gene (Verbakel, 1991) was subcloned into pTZ19R (Pharmacia), thereby generating the construction intermediate pUR2650 (Figure 1a),

in which the *Sty*I and *Hin*dIII sites used for the integration of the oligonucleotide cassette are unique.

For the assembly of the synthetic AFP gene, 50 pmol of each of the oligonucleotides, except AFL03 and AFP06, the ones at the 5' overlapping end of the cassette, were dissolved in 12 µl water, incubated for 2 min. at 95°C, and directly placed on ice. After this denaturation step, the oligonucleotides were phosphorylated in a final volume of 20µl, containing 2.5mM ATP, 5 mM DTT and about 10 U of polynucleotid-kinase, for 40 min. at 37°C, followed by a 2 min denaturation at 95°C and placement on ice. 10µl of each phosphorylated oligonucleotide was mixed with the corresponding DNA oligonucleotide to obtain duplex formation. After 2 min 95°C denaturation, each duplex was slowly cooled down to 30°C. Again 10µl of all five duplex mixtures were pooled and incubated in a final volume of 100µl, containing 50 mM Tris/HCl, pH 7.5, 8 mM MgCl2, 8 mM DTT, and 40 µg/ml gelatine and 10 U of DNA ligase, for two hours at 20°C. The ligation mix was then precipitated, and redissolved in 30 µl of TE-buffer. 15 µl of the mixture were placed on a 2 % agarose gel, and the DNA band of the expected size was cut out of the gel and finally purified through the gene clean II procedure, as recommended by the supplier. The obtained DNA fragment was then ligated into the *StyI/ Hin*dIII linearized vector pUR2650 and transformed into *E.coli* JM 109 by standard procedures, plasmid DNA of several transformants was isolated by the slightly modified alkaline-lysis mini-preparation method and analyzed by restriction analysis with several enzymes. This intermediate construct, containing the fusion cassette between α-galactosidase- and AFP-6 gene was named pUR2651 (see Figure 1b). The new 1.3 kb *Sac*I/*Hin*dIII fragment was than reintroduced into the *Sac*I/*Hin*dIII cut 2µ shuttle vector pUR2740 and transformed to *E.coli.* Several transformants were analyzed for the presence of the expected fragment by restriction analysis. From the presumably positive plasmids the correct assembly construction was confirmed by Sanger dideoxy sequencing of double stranded plasmids. This plasmid is designated pUR2652 (Figure 2a)

EXAMPLE 2 : Construction of pUR2653

By exchanging the DNA sequence coding for the α-mating factor against DNA coding for the anti freeze-peptide, we tried to obtain the mature AFP in the medium as a result of solely yeast borne proteolytic processing. For this purpose the six core-oligonucleotides (AFP01, AFP03, AFP05, AFP02, AFP04 and AFP06), comprising the major part of the AFP coding sequence as well as the double stop codon and the *Hin*dIII restriction site, were annealed to a new pair of oligonucleotides (AFL01 and AFL02), coding for an *Eag*I site, resp. an extra Ala at the N-terminus of AFP-6 and the first 4 amino acids (see SEQ. ID. NO: 3 and 4).

```
        AFL01                  AFP01
   EagI GGCC GAT ACT GCT TCT GAT GCC GCC GCT GCC GCT GCT TTG ACA GCT GCT
            CTA TGA CGA AGA CTA CGG CGG CGA CGG CGA CGA AAC TGT CGA CGA
                 AFL02                    AFP03
AAT GCT AAA GCC GCT GCT GAA TTG ACA GCT GCC AAT GCA GCC GCT GCT GCT
TTA CGA TTT CGG CGA CGA CTT AAC TGT CGA CGC TTA CGT CGG CGA CGA CGA
    AFP02                  AFP04                    AFP06

              AFP05
GCA GCT ACT GCG AGA TAAT A
CGT CGA TGA CGC TCT ATTA TTCGA HindIII
```

This synthetic genes simply generates an in frame transition between the coding part of AFP and the *Eag*I site at the end of the prepro-sequence in vector PSY16, a 2μm derived shuttle vector for leucine complementation, where the C-terminal end of the natural prepro α mating factor sequence, was changed in such a way, that ligation of DNA via EagI fuses the introduced coding part to the prepro-sequence. The DNA oligonucleotides were assembled into a double strand DNA fragment and purified as described in example 1 and the obtained 120 nucleotides long fragment was directly ligated into the *Eag*I/*Hin*dIII cut PSY16 vector. Some of the obtained transformants were grown up, the plasmid DNA isolated, and the construction controlled by restriction analysis and verified by dideoxy sequencing of the double stranded plasmids; This plasmid was designated pUR2653 (Figure 2b).

EXAMPLE 3: Construction of pUR2660, pUR2665, pUR2666 and pUR2667

To obtain a vector with more than one gene coding for AFP-6 we exchanged the oligonucleotide pair AFP05/AFP06 against a new set of oligonucleotides (AFL05/AFL06), comprising the C-terminus of the AFP and the four amino acid extension Lys, Arg, Glu, Ala, KREA, the proteolytic core part of the preproα mating factor spacer sequence, necessary to obtain mature N- and C terminal ends of the mating factor. The 3'-end oligonucleotides were designed in such a way, the they could be directly ligated into the *Eag*I site of pUR2653, thereby leaving only the new 5' end an intact *Eag*I restriction site, which then could be used for further additions of AFP cassettes.

```
      ⌜ AFL01              ⊤  AFP01                                    ⌝
EagI GGCC GAT ACT GCT TCT GAT GCC GCC GCT GCC GCT GCT TTG ACA GCT GCT
          CTA TGA CGA AGA CTA CGG CGG CGA CGG CGA CGA AAC TGT CGA CGA
           ⌞      AFL02                    ⊥
⌜                    AFP03           ⌝⌜
AAT GCT AAA GCC GCT GCT GAA TTG ACA GCT GCC AAT GCA GCC GCT GCT GCT
TTA CGA TTT CGG CGA CGA CTT AAC TGT CGA CGC TTA CGT CGG CGA CGA CGA
     AFP02     ⊥            AFP04                    ⌟⌞   AFL06

                   AFL05
GCA GCT ACT GCT AGA AAG AGA GA
CGT CGA TGA CGA TCT TTC TCT CTCCGG [EagI]
```

For that purpose the vector pUR2553 was linearized with *Eag*I, dephosphorylated by standard procedures, and the totally phosphorylated AFP cassette was ligated into the vector, transformed into *E.coli* JM109, several transformants analyzed as described and the correct assembly verified by DNA sequencing; the new recombinant plasmid was designed pUR2660 (Figure 3a).

Subsequent reassembly and reintroduction of the same oligonucleotide cassette into the unique *Eag*I site in the transition part between signal sequence and AFP coding part resulting in plasmids containing the DNA coding for 2 AFP's (pUR2660), 3 AFP's (pUR2665), 4 AFP's (pUR2666) and 5 AFP's (pUR2667). In each of these constructs every coding part is separated by the DNA coding for the KREA sequence (Figure 3a, 3b, 4a and 4b).

EXAMPLE 4: Construction of pUR2661

Trypsin is known to digest polypeptides carboxyterminal to amino acids Lys and Arg. Since amino acid sequence analysis revealed that Arg is the last amino acid of the mature AFP, digestion with this protease in principle allows the separation of AFP monomers from a given polyAFP peptide. But to leave the AFP in such a proteolytic digestion completely intact, the unique Lys has to be changed in the coding sequence. Structural analysis revealed, that this Lys is located on the more hydrophobic site of the molecule (Yang *et al.*, 1988, Davies *et al.*, 1990), a change into a neutral charged Ala further increases the amphiphilic character of the α helical structure. In extension of these options allowing the *in vivo* processing and maturation of poly-AFP precursor molecules, constructs were made, in which the unique Lys at amino acid position 18 within the AFP is exchanged against an Ala, the main amino acid of the AFP-6.

For the strongly related AFP-8 from winter flounder it was suggested, that amino acids Lys22 and Asp26 form a salt bridge, which is functional in stabilizing the α-helical configuration (Chakrabartty *et al.*, 1989). In a very recently published article, Mueller *et al.*, 1991, described a possible synergy between the C-terminal Arg and the salt bridge in AFP-8, whereby salt bridge and carboxyl Arg together do not affect the recrystallization inhibition activity, whereas either feature alone tends to reduce it.

Such a salt bridge seems also to be present in AFP-6, between lys18 and Glu22, which might have a similar stabilizing effect. The exchange of this Lys therefore not only allows the trypsin digestion of the direct fusion polypeptide, but also gives an idee about the contribution of this salt bridge in AFP-6 to the crystallization inhibition activity.

```
      ┌ AFL01            ┐┌   AFP01                                    ┐
EagI GGCC GAT ACT GCT TCT GAT GCC GCC GCT GCC GCT GCT TTG ACA GCT GCT
          CTA TGA CGA AGA CTA CGG CGG CGA CGG CGA CGA AAC TGT CGA CGA
          └      AFL02                  ┘└
5
┌                    AFP13          ┐┌
AAT GCT GCT GCC GCT GCT GAA TTG ACA GCT GCC AAT GCA GCC GCT GCC GCC
TTA CGA CGA CGG CGA CGA CTT AAC TGT CGA CGC TTA CGT CGG CGA CGG CGG
    AFP12     ┘└                   AFP04            ┘└  AFL08
10
                   AFL07             ┐
GGC TGC TAC TGC AAG AGA TAC TGC TAG C
CCG ACG ATG ACG TTC TCT ATG ACG ATC GCT ACGGCGGCGA
```

From the already described set of "core" oligonucleotides AFL01, AFL02, AFP01, AFP04 were used in combination with the newly synthesized oligonucleotides AFP12 and AFP13, which form a duplex displaying the central part of the AFP, with the mentioned Lys to Ala exchange at position 18. The second new pair of oligonucleotides AFL08 and AFL07 just served for the direct linkage of the coding sequences. The oligonucleotide pairs AFL01/AFL02, AFL07/AFL08, AFP05/AFP06, AFP01-/AFP12 were therefore used at a concentration of about 25 pmol, while the common structural parts of both copies, displayed by AFP01/AFP12 and AFP13/AFP04, were used at a final concentration of 50 pmol (Figure 6).The assembly was done as described, generating a *Eag*I/*Hin*dIII fragment with two directly linked APF coding parts, where in both genes the Lys codon was exchanged against Ala. This plasmid was named pUR2661 and is identical to pUR2660, with the exception of the 12 nucleotides coding for the KREA amino acid sequence and the mentioned Lys to Ala exchange.

EXAMPLE 5: Construction of pUR2674

Another possibility for increased AFP peptide expression in yeast is the repeated fusion of AFP-6 behind the carrier protein α-galactosidase, separated by internal spacers originating from the intervening sequences of the α-mating factor precursor molecule. To obtain a DNA template coding for 5 repeats of AFP-6 separated by KREA PCR technique was applied. Here, plasmid pUR2657 served as template during the reaction. The primers were designed in such a way, that an in frame

fusion between the end of the α-galactosidase gene at the *Sty*I site at the 3' end of the gene, -already used for the construction of pUR2652-, and the AFP coding part was insured. Both parts were again separated by the last 6 amino acids of the α-mating factor pro sequence and an additional extra alanine at the N terminus of the first AFP, which is a result of the introduction of the *Eag*I site for cloning purposes.

```
              P   R   S
     5' CCC CCA AGG TCT AAA AGA GAG GCT GAA GCG GCC      pAFC01
          StyI
pUR2667    3'
           5'
```

TTT TCT CTC CGA CTT CGC CGG 5'

AAA AGA GAG GCT GAA GCG GCC GAT ACT 3'

K R E A E A A D T

The corresponding primer at the 3' end of the gene was synthesized in such a way, that a unique hybridization on the template was ensured, thereby leaving the *Hin*dIII site behind the stop codon intact.

```
                                HindIII
primer AFC02              CGC TCT ATT ATT CGAATAATA   5'
pUR2667          5' ACT GCG AGA TAA TAA GCTTATTATCTCGC 3'
                 3' TGA CGC TCT ATT ATT CGAATAATAGAGCG 5'
                     T   A   R
```

PCR amplification of the template was carried out in a Perkin Elmer Cetus DNA Thermal Cycler. The reaction was carried out in 100 µl 10 mM Tris, HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, with 0.2 mM of each dNTP, 100 pmol of the DNA oligonucleotides KLM06 and KLM09, about 0.1 µg of *Eco*RI digested DNA and 1 U of Amplitaq polymerase. Incubation parameters were set as follows: 32 cycles/ 1 min 95 °C/ 2 min 50 °C/ 2,5 min 72 °C. About 20µl of the reaction volume was placed on a 1% agarose gel, the DNA fragment of about 650 bp isolated and purified with the GenecleanII kit (Bio 101 Inc.) according to the manufacturers protocol. The fragment was subsequently digested with *Sty*I and *Hin*dIII and ligated into the *Sty*I/*Hin*dIII digested plasmid pUR2650. After transformation into *E.coli* JM109 the new plasmid was isolated and digested with *Sac*I/*Hin*dIII. The about

1.85 Kbp large fragment comprising the DNA coding for the SUC2 signal sequence and the α-galactosidase (5x)AFP fusion was subsequently ligated into the vector pUR2741, whereby plasmid pUR2741 is a derivative of pUR2740 (Verbakel, 1991), where the second *Eag*I restriction site in the already inactive Tet resistance gene was deleted through *Nru*I/*Sal*I digestion.

One of the positive clones, designated pUR2674 (Figure 5), was further characterized, the DNA isolated and purified according to the Qiagen protocol and subsequently characterized by DNA sequencing. DNA sequencing was mainly performed as described by Sanger, and Hsiao, here with the Sequenase version 2.0 kit from United States Biochemical Company, according to the protocol with T7 DNA polymerase (Amersham International plc) and [^{35}S]dATPαS (Amersham International plc: 370 MBq/ml; 22 TBq/mmol).

EXAMPLE 6: Construction of pUR2676, pUR2677, pUR2678 and pUR2679

Another option for the tandem expression of AFP's in yeast is the direct fusion of AFP coding part to each other, without altering the coding sequence. Here again the unique presence of Arg at the C-terminal position mature AFP-6 is Arg, allows a post-fermentative digestion of the fusion product into single monomers. This can be obtained through either the addition of carboxypeptidase Arg directly to the yeast culture supernatant or the partially or completely purified culture medium.

Therefore again PCR technique was applied. Through the use of 2 corresponding primers, complementary to both ends of the AFP-6 coding sequence, DNA fragments were generated, encoding only structural information of AFP. For the direct linkage of the PCR fragment to the first copy already present in pUR2653, the *Pst*I site present in the 3'-part of the AFP coding sequence was used. Therefore, the 5' PCR oligonucleotide AFPPR1JVA also enclosed the DNA sequence coding for C-terminal part of AFP from the *Pst*I site on. The corresponding 3' PCR oligonucleotide AFPR2JVA contained a sequence extension at the 5' end comprising a *Stu*I starting with the last codon of the AFP gene, to ensure the direct in frame blunt end linkage between different AFP copies (see SEQ. ID. NO: 15 - 20).

PCR primer AFPPRIJVA:

```
        pUR2653              5'  GAT ACT GCT TGT GAT GCC GCC GCT GCC
                             3'  CTA TGA CGA ACA CTA CGG CGG CGA CGG
        PstI                     ||| ||  ||  ||| ||| ||| ||| ||| |||
5' CCCCC CTGCA GCT ACA GCT AGG GAT ACC GCC TGT GAT GCC GCC GCT GCC
               A   T   A   R   D   T   A   S   D   A   A   A   A
```

PCR primer AFPR2JVA:

```
                                          StuI    HindIII
            CGA CGA CGA CGC CGA TGA CGC TCC GGA ATTCGAACGTAC 5'
            ||| ||| ||| ||  ||| ||| ||| ||
5'      GCC GCT GCT GCT GCA GCT ACT GCG AGA
3'      CGG CGA CGA CGA CGT CGA TGA CGC TCT
            A   A   A   A   A   T   A   R
```

Primer AFPR2JVA also inactivated the *Pst*I site present in the template, without altering the amino acid sequence. This allowed the *Pst*I/*Hin*dIII digestion of the PCR product, which was generated and purified as described earlier. To facilitate further cloning procedures, the vector pUR2650 was digested with *Eag*I/*Hin*dIII, the larger, about 3.1 kbp vector fragment isolated, and subsequently ligated with the 0.1 kbp long *Eag*I/*Hin*dIII fragment of pUR2653, which comprised the AFP coding sequence, thereby generating an construction intermediate with an unique *Pst*I site in the 3'-coding part. The vector was digested with *Pst*I/*Hin*dIII, the about 3.2 kbp fragment purified and ligated with the *Pst*I/*Hin*dIII digested PCR product. After transformation into *E.coli* JM109 the plasmid was isolated and sequenced as described earlier. The complete about 0.25 kbp *Eag*I/*Hin*dIII fragment containing the two directly linked AFP coding parts, was then exchanged against small *Eag*I/*Hin*dIII fragments in pUR2652 and pUR2653. Both plasmids, denoted pUR2676 and pUR2677, were sequenced as described to confirm the accurate assembly of the fragments (see Figure 7a and 8a).

To further expand the AFP copy number in the plasmid, a second PCR reaction can be executed, based on plasmid pUR2676 as template. Beside the oligonucleotide AFPJ2JVA, which automatically creates a *Stu*I site behind the AFP coding sequence, a new 5' PCR oligonucleotide was synthesized, which allows the generation of a DNA fragment coding for two directly joined AFP coding parts, with an additional *Hin*cII site overlapping with the first codon of the first AFP copy.

```
                                        D   T   A   S   D   A   A   A   A
                pUR2676          5'   GAT ACC GCC TGT GAT GCC GCC GCT GCC
                                 3'   CTA TGG CGG ACA CTA CGG CGG CGA CGG
                                      ||  ||  ||  ||| ||| ||| ||| ||| |||
AFPR3JV    5'   AAACCGAATTC GTC GAC ACC GCC TGT GAT GCC GCC GCT GCG
                 EcoRI     HincII
```

After PCR this DNA fragment could be isolated and purified as described, digested with *Eco*RI and *Hin*dIII and cloned into one of the commercially available usual *E. coli* cloning plasmids, eg. pTZ19R ex Pharmacia. From this plasmid the about 0.25 kbp *Hin*cII/*Stu*I fragment can be isolated and ligated behind the unique *Stu*I site present at the end of the second AFP coding sequence in pUR2676 and pUR2677 respectively, thereby producing the plasmids pUR2678 and pUR2679 (Figure 7b and 8b). This ligation can be repeated several times, thereby increasing the in each ligation round the copy number behind either the SUC2 sequence or the prepro-α-mating factor sequence with two.

EXAMPLE 7: Measurement α-galactosidase activity of pUR2652 *S. cerevisiae* transformants

From the Qiagen purified plasmid DNA of pUR2652, containing the α-galactosidase-AFP fusion under the control of the inducible GAL7 promoter and behind the invertase signal sequence about 5 µg was transformed to *S. cerevisiae* SU10 according to the method of Beggs *et al.* 1978. and plated onto YNB-plates containing histidine and uracil. Several of this transformants were incubated overnight in a 4 ml culture YNB, with 2 % glucose, ura and his. To test the induction of expression about 400µl of the overnight cultures were used to inoculate a 20 ml culture YP medium, containing 5 % galactose. After incubation at 30 °C for about 40 hours, the A660 was determined, the culture was centrifuged for 5 min with 4000 rpm and the α-galactosidase activity in the supernatant was measured.

α-galactosidase activity was determined at 37°C using p-nitrophenyl α-D-galactopyranoside (PNPG, ex Sigma) as substrate in a 0.1 M sodium acetate buffer, pH 5.0. A solution of 22.22 mM PNPG, in acetate buffer was freshly prepared and 0.1 ml of an 1:1000 in acetate buffer diluted supernatant solution was added to 0.9 ml pre-

warmed substrate solution. The mixture was incubated in a thermostatically controlled water bad for 5-10 minutes, and then stopped by addition of 2 ml 10% sodium carbonate solution. A blank was made as described below, by adding just 0.1 ml sodium acetate. The concentration of p-nitrophenolate was determined at 405 nm at room temperature. The difference between the molar absorptivity of p-nitrophenolate and P-nitrophenyl-α-galactopyranoside at 405 nm and pH12 was determined experimentally as 18.160 $cm^2/\mu mol$. Therefore the enzyme activity was calculated with the help of the following formula: {(At -Ab)/t} * 1.652 µmol per ml, where At is the absorbance at the incubation time t in minutes, and Ab the absorbance of the blank. One Unit was defined as the hydrolysis of a µmol of substrate in 1 min at 37°C and at a pH of 4.5. The specific activity of guar α-galactosidase is 100 U per mg protein.

For the quantitative assay of the α-galactosidase enzyme in the culture medium of *S. cerevisiae* SU10 cells containing pUR2652 different dilutions were used. The expression level was calculated as mg α-galactosidase/L culture medium. As controls served a SU10 strain transformed with pUR2740, the parental plasmid without the AFP cassette and SU10 untransformed, to correct for the natural MEL1 expression level. A concentration of about 300 mg/L culture fluid was determined for different SU10 α-gal/AFP transformants, compared to 6 mg/l for the untransformed SU10 and approximately 300 mg/l for the SU10 with pUR2740, indicating the same amount of expression and secretion for the fusion protein as for α-galactosidase. Since both enzymatic measurements were based on the same amount of protein present, this also indicates an identical specific α-galactosidase activity for the fusion construct.

EXAMPLE 8. SDS-PAGE protein gelelectrophoresis and Western analysis of cell and culture fluid of SU10 cells containing either pUR2652 or pUR2653

48 hours incubated cultures were centrifuged for 5 min at 4000 rpm and cells and supernatant were collected at stored at -20 °C. The cells were broken with glass beads and after isolation the cell-extract was centrifuged in an Eppendorf centrifuge for 5 min. The soluble cell fraction supernatant was stored at -20 °C. Denaturating

SDS polyacrylamide gel electrophoresis to separate the proteins was performed mainly as described by Laemmli, 1970. The Western blot analysis of the SDS-PAGE electrophoresis was performed principally as described by Towbin *et al.*, 1979, by semi dry blotting. After blotting the Immobilon-P membrane was blocked with 5 % skimmed milk in 150 mM NaCl, 50 mM Tris/HCl pH 7.4 and afterwards incubated in 1 % skimmed milk in 150 mM NaCl, 50 mM Tris/HCl, 0.1 % Tween 20, pH 7.4 and antiserum against AFP-6 or α-galactosidase respectively. Both antisera were raised by immunization of rabbits with α-galactosidase purified from guar, and with AFP-6 obtained by Merriefield synthesis. Incubation was performed overnight at room temperature under soft agitation. Unabsorbed antibodies were removed by washing with the incubation buffer (3x 5 min) The membrane was incubated with the second antibody (Goat anti-rabbit IgG (H+L), Horseradish Peroxidase Conjugate; Biorad, Richmond) for 2 hours at room temperature. The enzyme was developed using 4-chloro-1-naphthol. For the transformant containing pUR2652 Western analysis with AFP-6 antisera revealed specific signals (see Figure 8b). Here, specific signals were detected within the soluble fraction of the yeast cell and in the culture fluid, whereby in both cases the apparent size of the obtained fusion-protein was about 46 kD, which is a little bit larger than α-galactosidase expressed in yeast (Overbeeke *et al*, 1987), and in good agreement with the result expected, due to the of nearly 4 kD structural information. Antiserum against α-galactosidase reacted specifically with the same protein bands, thereby verifying the expression of the α-galactosidase AFP fusion protein in yeast (data not shown). Both PAA Coomassie stain and immune specific stain revealed some minor degradation products.

A somehow different situation was observed with the SU10 cells transformed with pUR2653. Here, neither in the Coomassie stain nor in the immune specific stain any signal could be detected within the medium (see Figure 9a and 9b), while the Western analysis of soluble cell fraction revealed two bands, one of them a cross-reacting host protein, which is also present in the untransformed yeast (Figure 9b, lane "c"), while the second, significantly smaller signal was only obtained in transformed yeast cells, presumably presenting the AFP. Whether the absence of AFP in the culture medium was the result of inhibition of secretion or rapid degradation remains to be elucidated.

EXAMPLE 9. Thermal hysteresis assay with culture fluid of cells containing of pUR2652 and pUR2653

In principle, this assay measures the difference between freeze- and melting point (de Vries, 1983) The range, in which this effect is measured is around 1 degree centigrade. In the described experiment substances were dissolved in water, soaked through capillary forces into micro-slides and quickly frozen. The samples were then placed into a controlled thermal ethanol bath at a temperature where the initial crystal nucleus is stable (visually controlled with Olympus stereo microscope at a 15 fold magnification). The temperature was then stepwise decreased with 0.1°C/min until the crystals started growing, this was defined as crystallization temperature. This process was continued for 2 minutes, and than the temperature was slowly increased in the same intervals. As soon as visually detectable melting occurred, the melting temperature was reached. As a positive control a sample chemically synthesized winter flounder peptide was used, -produced by Merriefield synthesis-, at a final concentration of 3% , while α-galactosidase, the carrier protein in the fusion situation served as negative control. The results are graphically summarized in Figure 10. The difference between both control substances was significant, hence, a difference of 0.1°C in freeze and melting point was considered as inactivated, compared to the 0.7 degree for the chemically synthesized AFP.

The concentrated culture fluid of cells carrying the fusion gene expressed by pUR2652 revealed a hysteresis of 0,5 degree, which is similar to the 3% solution chemically synthesized AFP.

The effect obtained with the culture fluid of cells with plasmid pUR2653 was with a thermal hysteresis of 0.8°C at the same level than the pure 3% AFP solution.

EXAMPLE 10. Recrystallization assay of yeast cells containing pUR2652

1µl of solutions containing 30% sucrose and either 3% of pure chemically synthesized AFP or approx. 40% of freeze dried supernatant of culture fluid of yeast cells containing pUR2652 -final concentration- were placed onto microscope slides, covered with BDH 16 mm slides and sealed. The sample was set on a Linkam THMS coldstage, connected with a Linkam CS 196 cooling system and controlled by a Linkam TMS 91 controller. The stage was subsequently crash cooled

(δ 99 °C/ min.) to -40 °C to produce a large amount of small sized ice crystals. The size of the ice crystals in the solution was recorded by a video camera. The stage was then rapidly raised to -5 °C and kept there for 1 hour. The growth of the ice crystals was followed visually and again registered by video recording. While the solution bearing no additives displayed the foreseen large crystals after incubation 9 (Figure 11a), the sample containing 3% AFP remains generally unchanged compared to the starting conditions, clearly indicating the growth inhibiting effect of AFP (see Figure 11b). The solution containing about 40% of the freeze dried supernatant of pUR2652 displayed a different picture. Here, the crystals appeared to be significantly smaller and more needlelike (see Figure 11c), indicating the even a fusion construct comprising not more than about 5% structural information of AFP is capable of restraining ice crystal growth.

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* * * * *

CLAIMS

1. A process for producing peptides inhibiting ice crystal growth, so-called anti-freeze peptides (AFP), by expressing in a host organism a gene encoding an AFP or a fusion protein comprising an AFP, which comprises
(i) forming a DNA construct comprising in the order given
(a) a strong, optionally inducible, promoter active in the host organism,
(b) an ATG start codon, that may be present in an optional DNA signal sequence capable of secreting the protein produced by the host organism during expression of an AFP-encoding gene of (c) below, which signal sequence can be homologous or heterologous to the AFP-encoding gene and is in reading frame with the ATG start codon,
(c) at least one desired AFP-encoding gene in reading frame with the ATG start codon, and
(d) a stop codon bound to the 3' end of the AFP-encoding gene,
(ii) transforming a host organism with the DNA construct of (i) in such a way that the host organism can express at least the AFP-encoding gene,
(iii) growing the transformed host organism under conditions whereby the expression of at least the AFP-encoding gene occurs or is induced, and
(iv) optionally collecting the produced AFP or fusion protein, or peptide or protein obtained therefrom by further processing.

2. A process according to claim 1, in which the promoter is an inducible GAL7 promoter or a constitutive GAPDH promoter of *Saccharomyces cerevisiae*.

3. A process according to claim 1, in which the DNA signal sequence is the DNA pre-sequence of the α-mating factor of *S. cerevisiae*.

4. A process according to claim 3, in which the DNA construct also comprises the pro-sequence of the α-mating factor of *S. cerevisiae* between the pre-sequence and the AFP-encoding gene, whereby the pre-sequence, the pro-sequence and the AFP-encoding gene are in the same reading frame.

5. A process according to claim 1, in which the host organism is a yeast.

6. A process according to claim 1, in which the DNA construct contains a fused gene comprising at least part of the α-galactosidase gene of *Cyamopsis tetragonoloba* and at least one AFP-encoding gene with its 5' end in-frame-fused to the 3' end of the at least part of the α-galactosidase gene.

7. A process according to claim 6, in which the DNA construct contains the invertase signal sequence of *S. cerevisiae* preceding the fused gene.

8. A process according to claim 1, in which the connection between two or more genes comprises a DNA sequence encoding a processing site that can be used to split off at least one AFP from the remainder of the protein, either during or after secretion of the fusion protein or another precursor-AFP.

9. A process according to claim 1, in which the "at least one AFP-encoding gene" comprises a tandem-repeat of one or more AFP-encoding genes, whereby optionally one or more AFP-encoding genes can be separated by a DNA sequence encoding a processing site.

10. AFP prepared by a process according to any one of the previous claims.

11. Use of an AFP as claimed in claim 10 for inhibiting ice crystal growth.

12. A yeast having improved freeze tolerance, containing and/or surrounded by AFP produced by a process as claimed in claim 1.

13. Use of a yeast as claimed in claim 12 in a process for preparing frozen dough or in compositions that can be frozen.

* * * * *

Fig.1A.

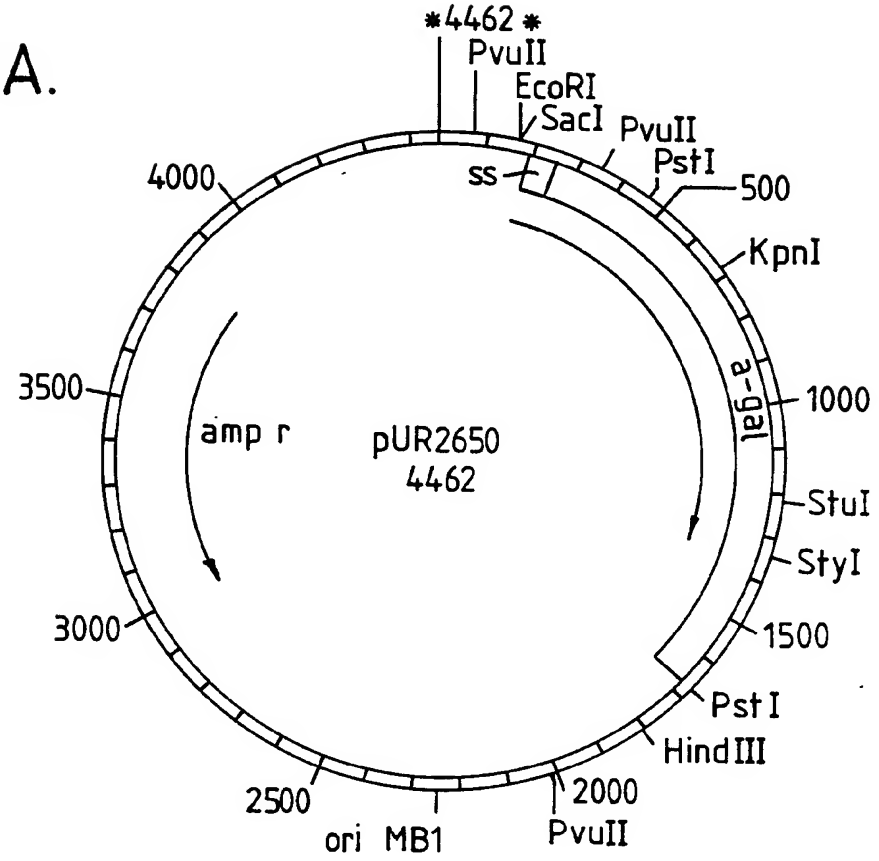

4462
PvuII
EcoRI
SacI
PvuII
PstI
500
KpnI
1000
StuI
StyI
1500
Pst I
HindIII
2000
PvuII
2500
ori MB1
3000
3500
4000
ss
amp r
pUR2650
4462
a-gal

Fig.1B.

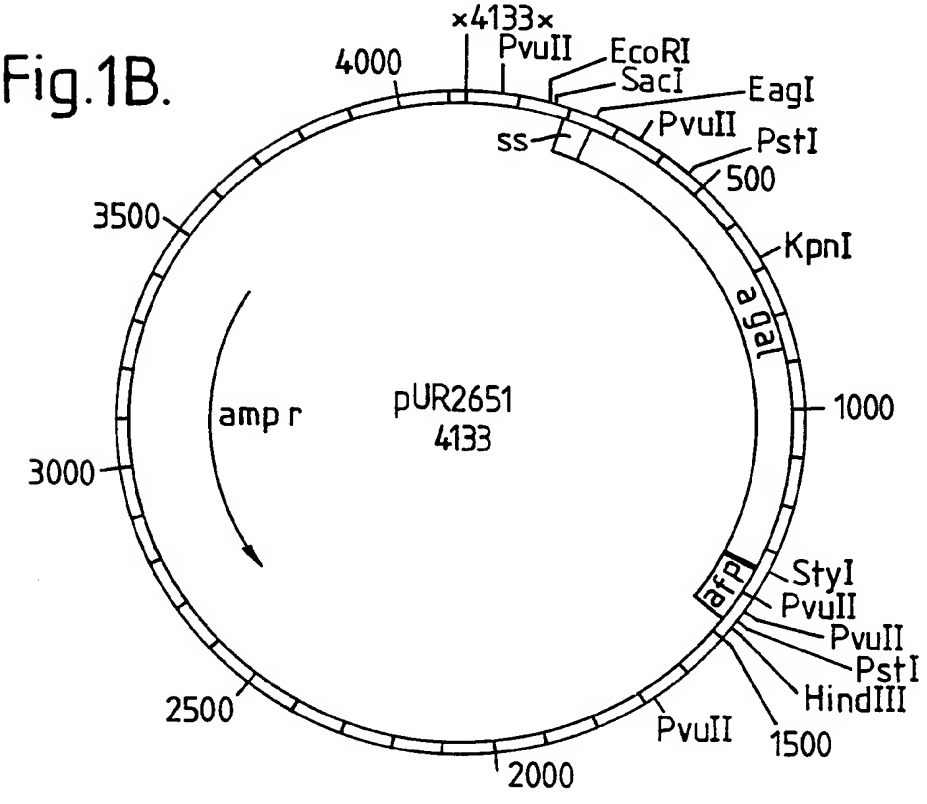

×4133×
PvuII
EcoRI
SacI
EagI
PvuII
PstI
500
KpnI
1000
StyI
PvuII
PvuII
PstI
HindIII
1500
PvuII
2000
2500
3000
3500
4000
ss
amp r
pUR2651
4133
a gal
afp

Fig. 2A.

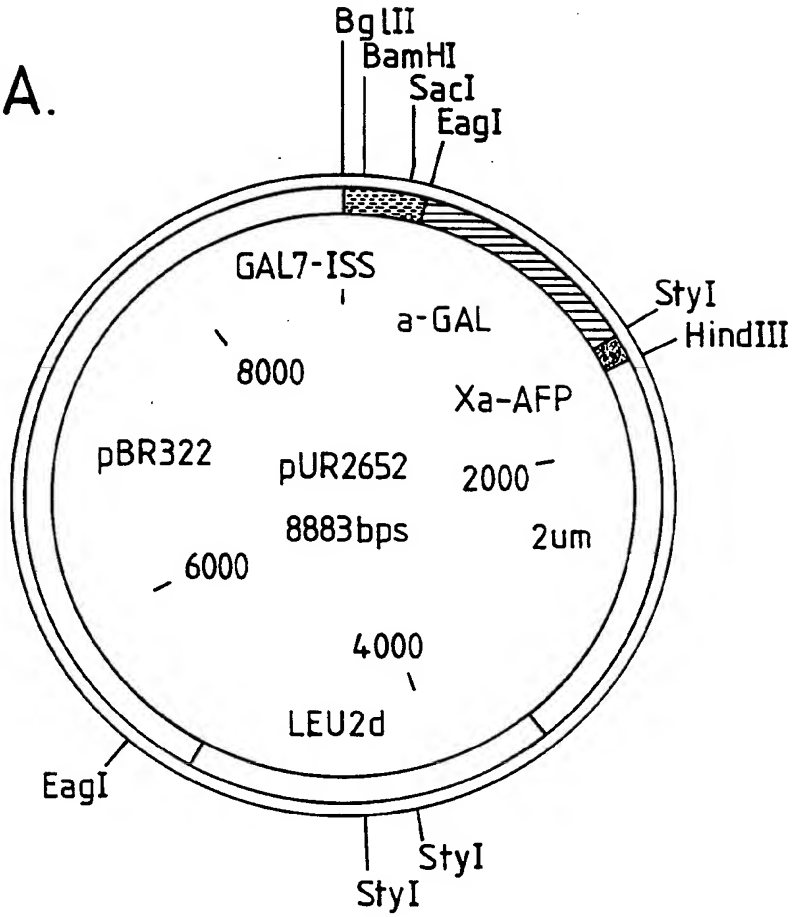

BglII
BamHI
SacI
EagI
GAL7-ISS
a-GAL
StyI
HindIII
8000
Xa-AFP
pBR322
pUR2652
2000
8883bps
2um
6000
4000
LEU2d
EagI
StyI
StyI

Fig. 2B.

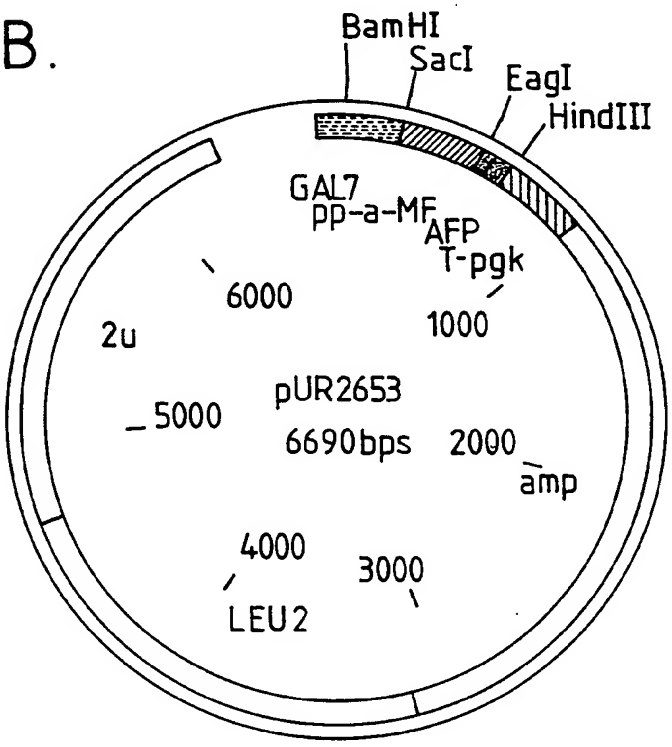

BamHI
SacI
EagI
HindIII
GAL7
pp-a-MF
AFP
T-pgk
6000
1000
2u
pUR2653
5000
6690bps
2000
amp
4000
3000
LEU2

Fig.3A.

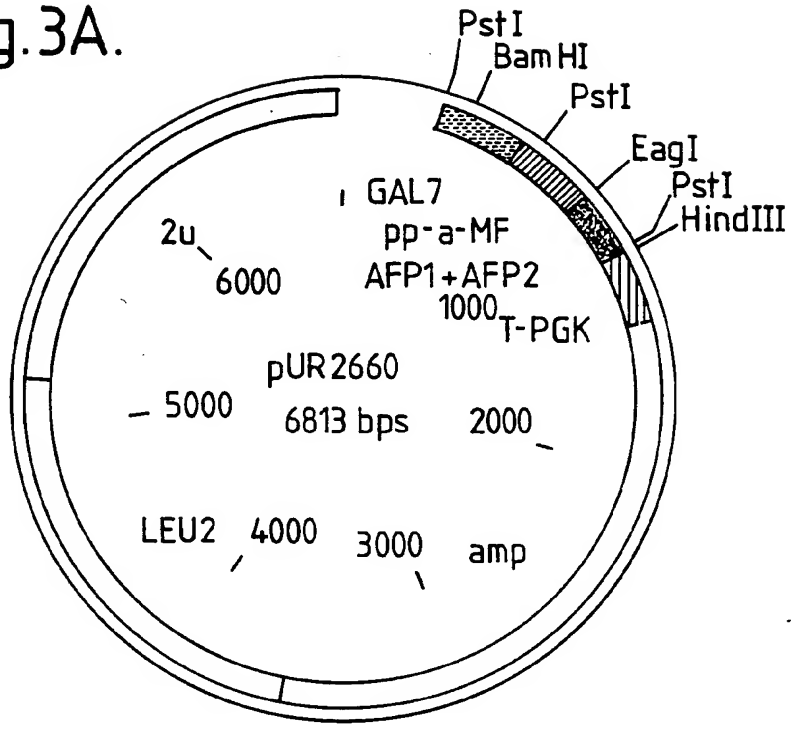

PstI
BamHI
PstI
EagI
PstI
HindIII
GAL7
pp-a-MF
AFP1+AFP2
T-PGK
2u
6000
1000
pUR2660
5000
6813 bps
2000
LEU2
4000
3000
amp

Fig.3B.

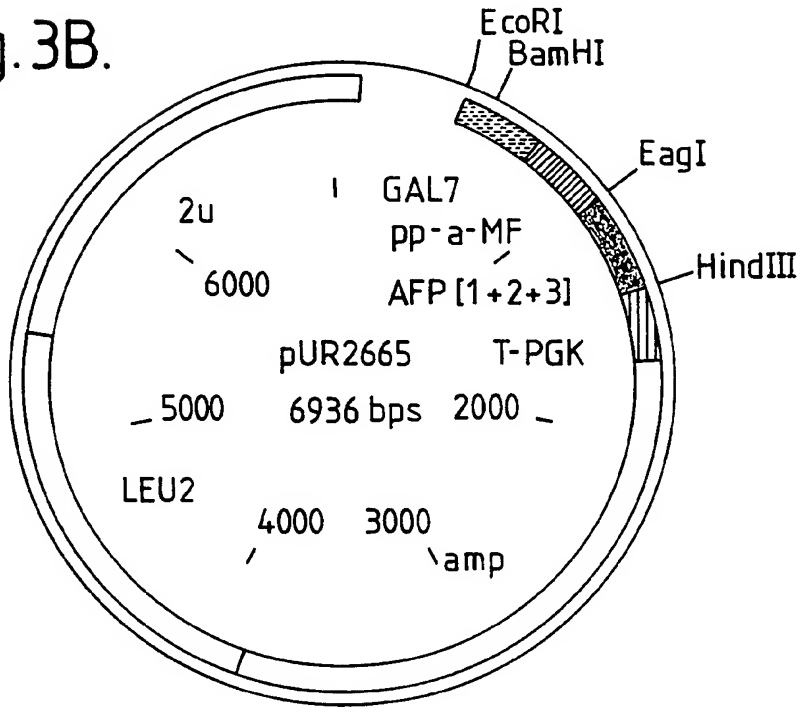

EcoRI
BamHI
EagI
HindIII
GAL7
pp-a-MF
AFP [1+2+3]
2u
6000
pUR2665
T-PGK
5000
6936 bps
2000
LEU2
4000
3000
amp

Fig.4A.

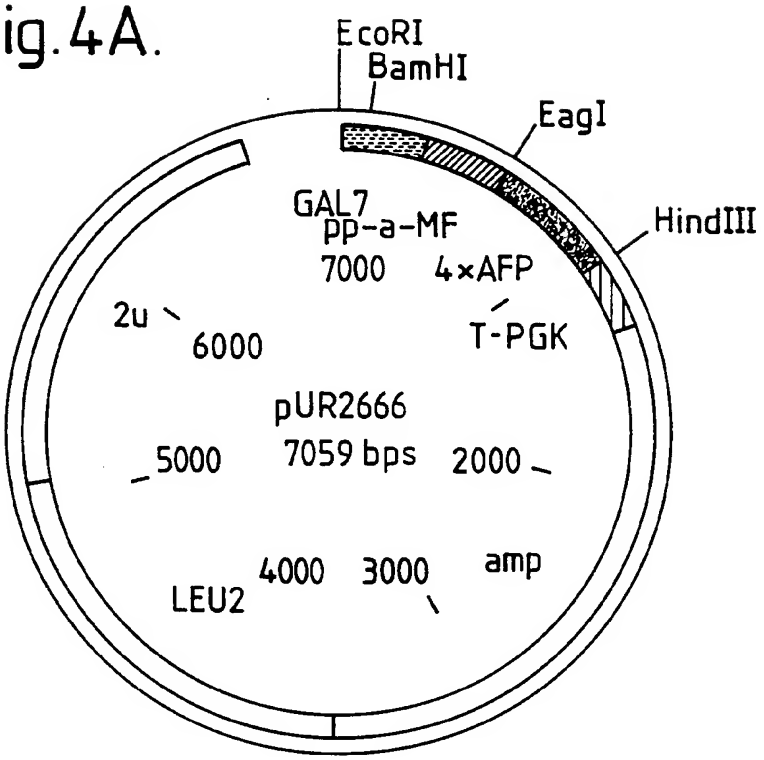

EcoRI
BamHI
EagI
HindIII
GAL7
pp-a-MF
7000
4×AFP
T-PGK
2u
6000
pUR2666
7059 bps
5000
2000
4000
3000
amp
LEU2

Fig.4B.

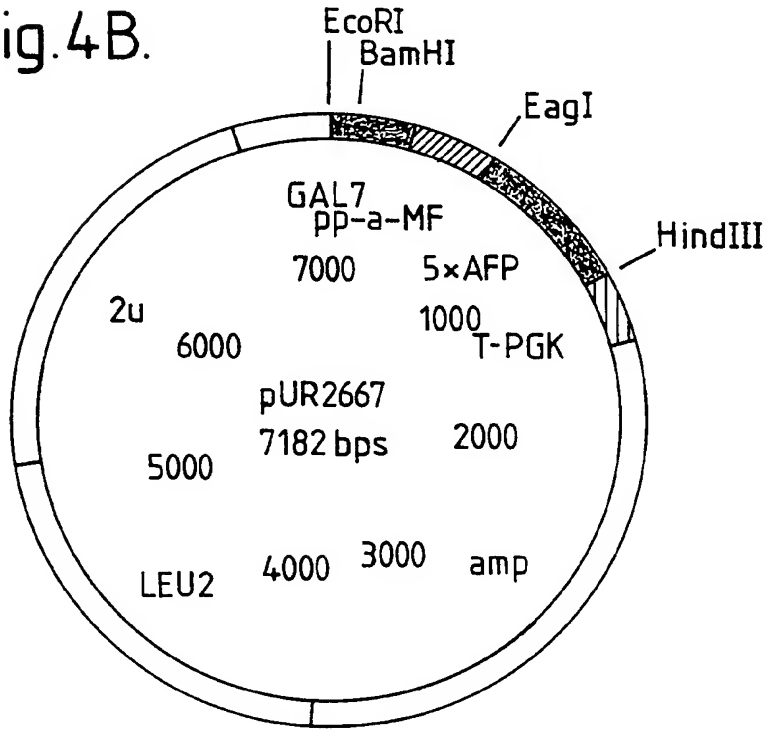

EcoRI
BamHI
EagI
HindIII
GAL7
pp-a-MF
7000
5×AFP
1000
T-PGK
2u
6000
pUR2667
7182 bps
2000
5000
4000
3000
amp
LEU2

Fig.5.

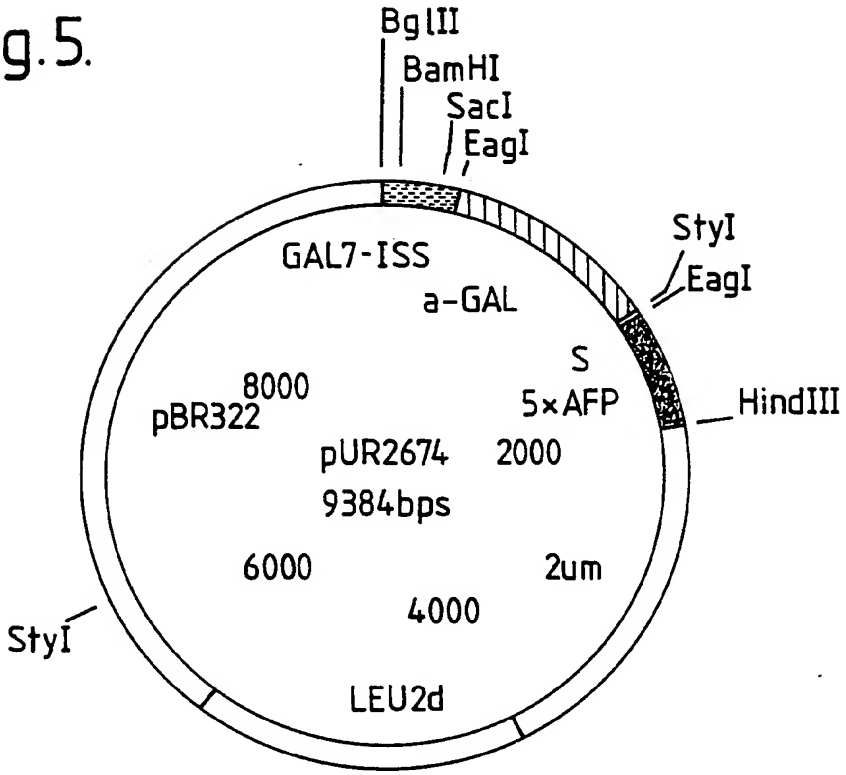

BglII
BamHI
SacI
EagI
StyI
EagI
GAL7-ISS
a-GAL
S
5×AFP
HindIII
8000
pBR322
pUR2674
2000
9384bps
6000
2um
4000
StyI
LEU2d

Fig.10.

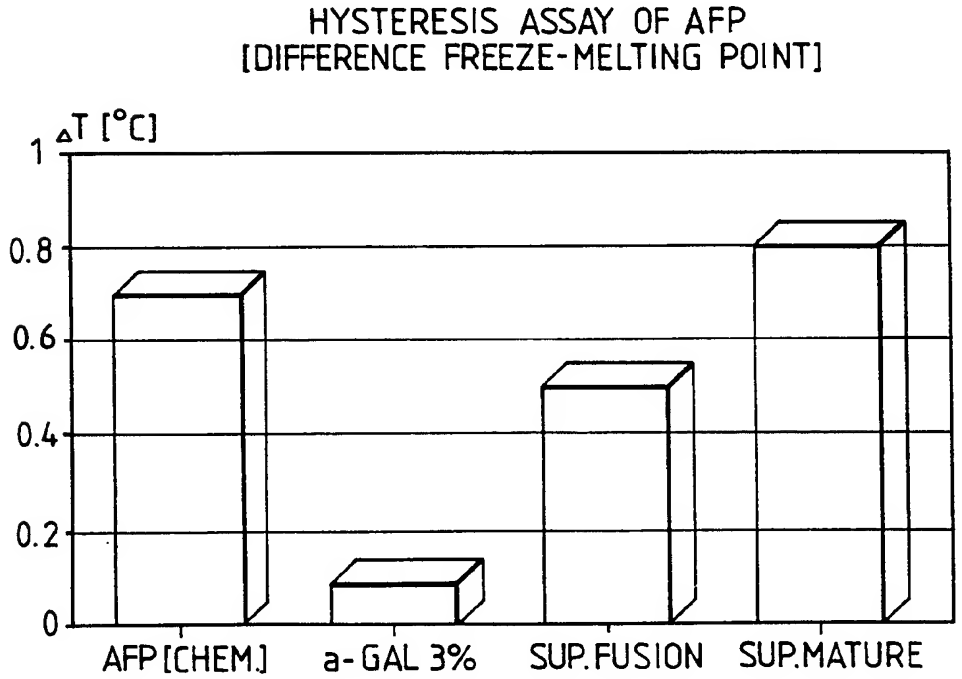

HYSTERESIS ASSAY OF AFP
[DIFFERENCE FREEZE-MELTING POINT]
ΔT [°C]
1
0.8
0.6
0.4
0.2
0
AFP [CHEM.]
a-GAL 3%
SUP. FUSION
SUP. MATURE

Fig. 6A.

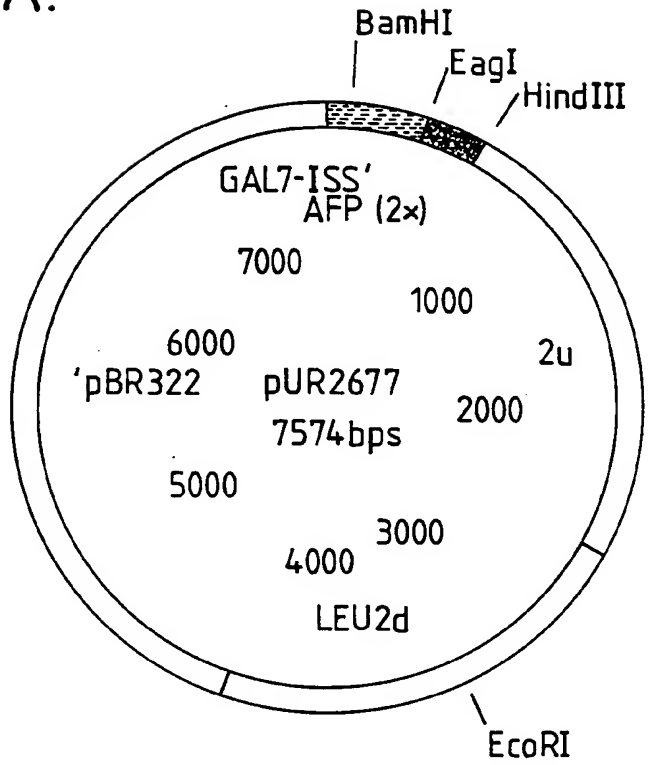

BamHI
EagI
HindIII
GAL7-ISS'
AFP (2x)
7000
1000
6000
2u
'pBR322
pUR2677
2000
7574bps
5000
3000
4000
LEU2d
EcoRI

Fig. 6B.

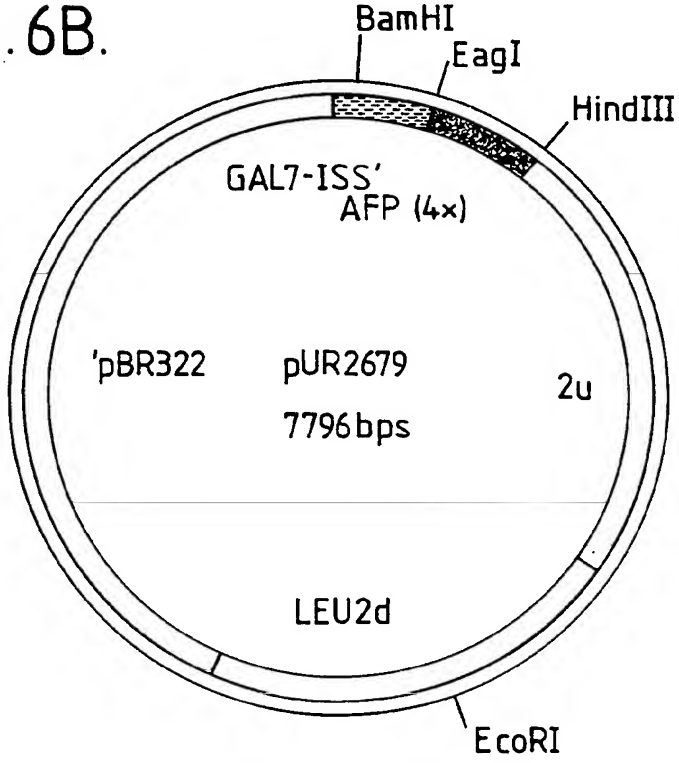

BamHI
EagI
HindIII
GAL7-ISS'
AFP (4x)
'pBR322
pUR2679
2u
7796bps
LEU2d
EcoRI

Fig.7A.

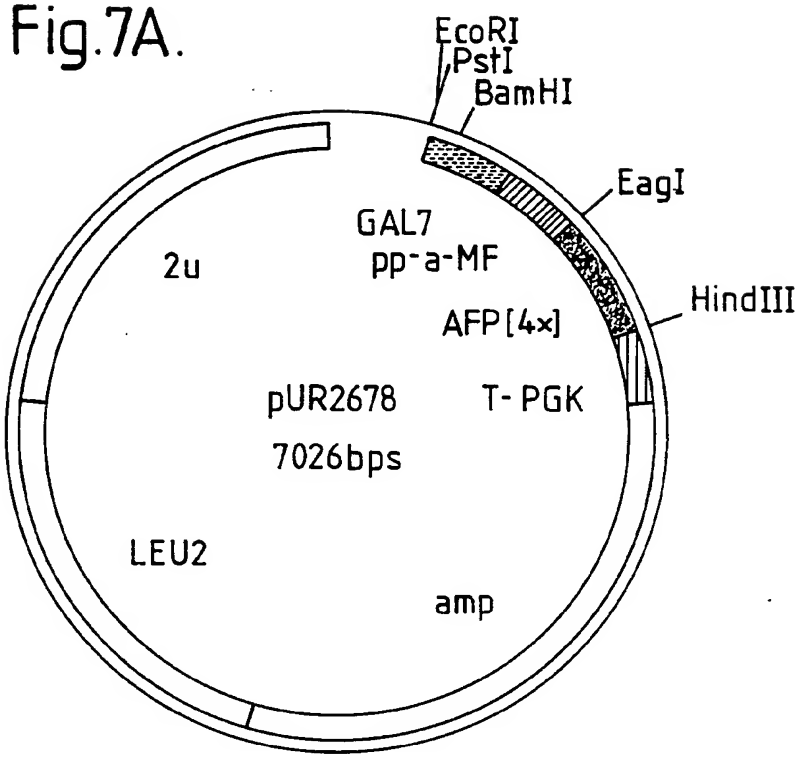

EcoRI
PstI
BamHI
EagI
HindIII
GAL7
pp-a-MF
2u
AFP[4x]
pUR2678
T-PGK
7026bps
LEU2
amp

Fig.7B

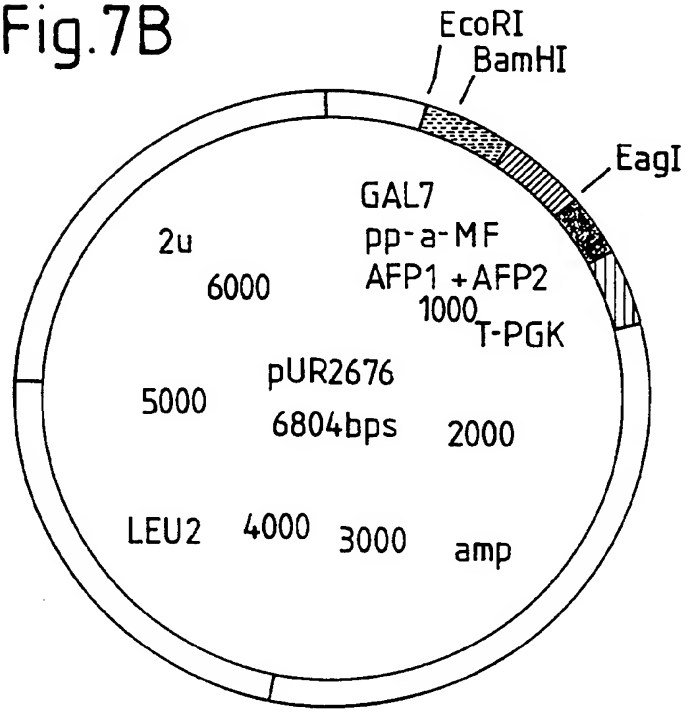
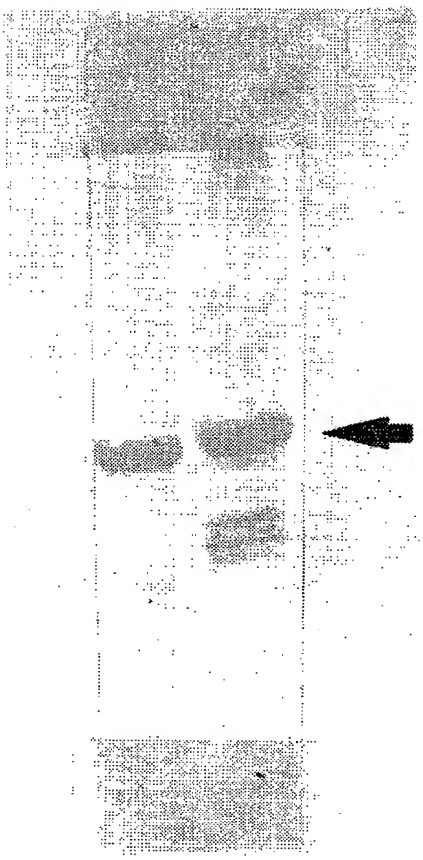
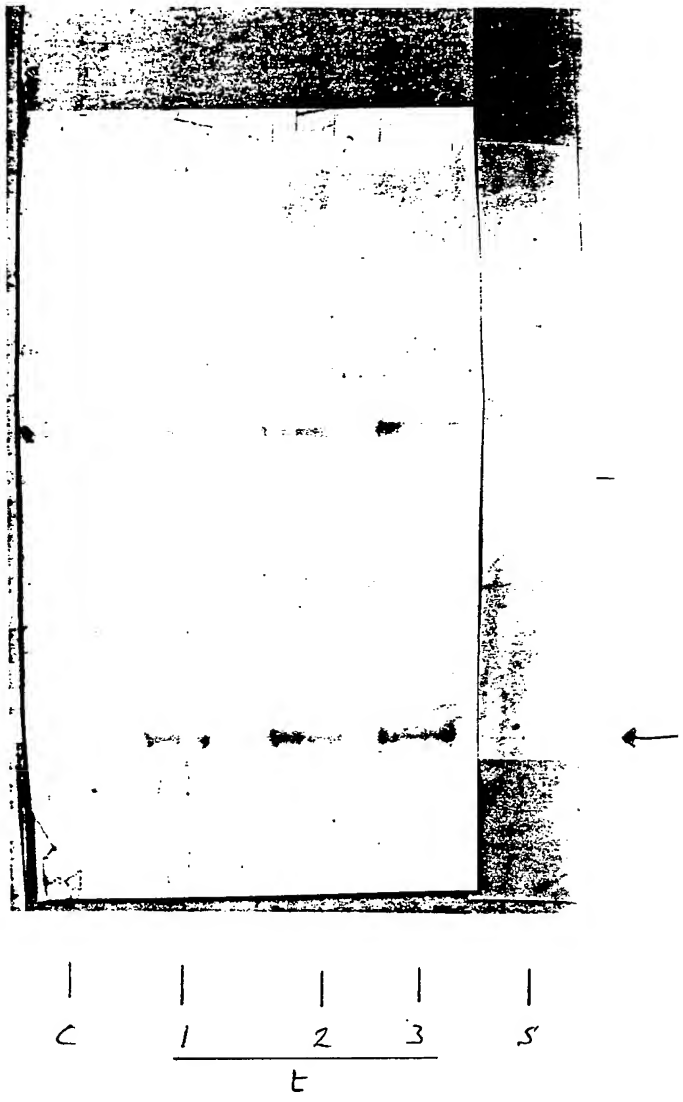

EcoRI
BamHI
EagI
GAL7
pp-a-MF
2u
6000
AFP1 +AFP2
1000
T-PGK
pUR2676
5000
6804bps
2000
LEU2
4000
3000
amp

Fig. 8. (2/2)



b) Immuno specific stain

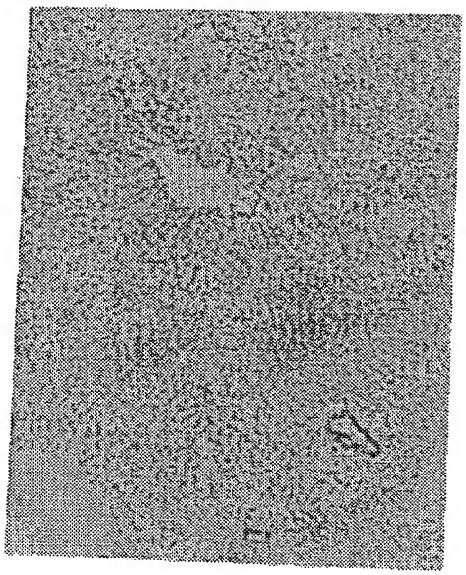
Fig. 9. (2/2)



b) Immuno specific stain

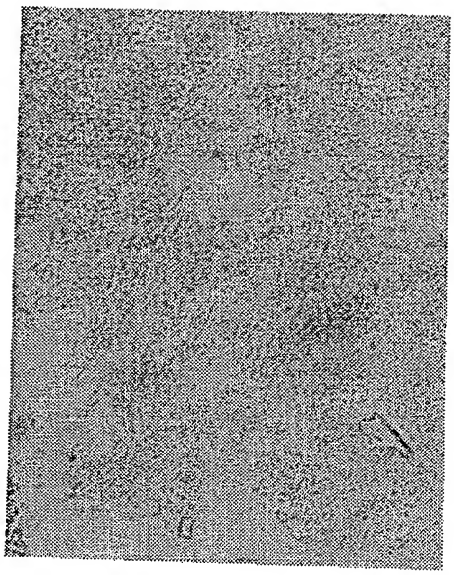
Fig. 11.

(3/6)



B.1 3% AFP-6 in 30% sucrose
T = 0 (before incubation at -5°C)

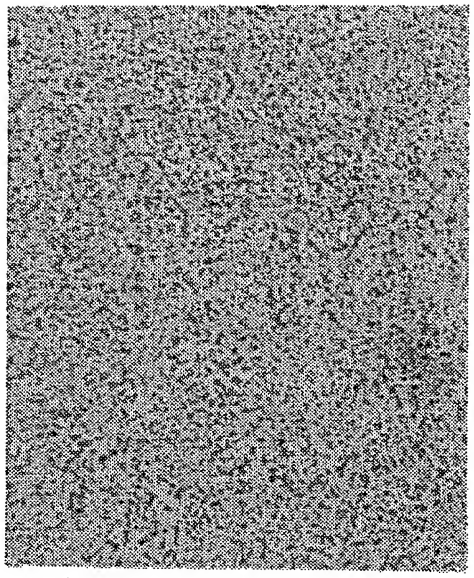
(4/6)



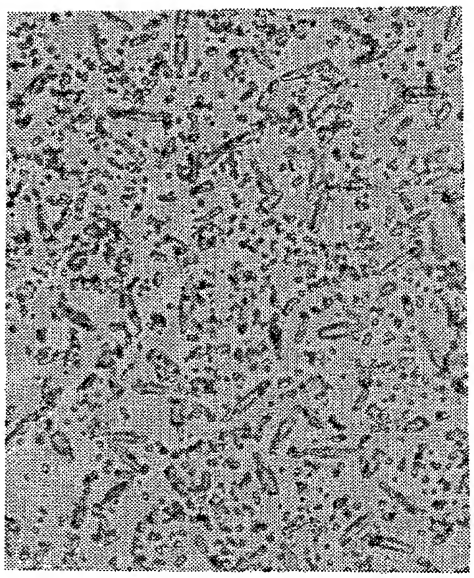
B.2 3% AFP-6 in 30% sucrose
T = 60 (after incubation at -5°C)

Fig. 11.

(5/6) (6/6)



C.1 pUR 2652 at −15°C.
T = 0 (before incubation)



C.2 pUR 2652 at −15°C.
T = 60 (after incubation)

INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/EP 93/01969

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/81 C12N15/62 C12N15/56 C07K13/00 C12N15/12
C12N1/19 A21D8/04 C12N9/40 //(C12N1/19,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 13571 (DNA PLANT TECHNOLOGY CORPORATION) 15 November 1990 cited in the application see page 21, line 18 - line 26; examples 11,12	1,5, 10-13
Y	---	2,6-8
Y	PHD THESIS . RIJKSUNIVERSITEIT 1 May 1991 , UTRECHT, THE NETHERLANDS pages 51 - 120 VERBAKEL J.M.A. 'Heterologous gene expression in the yeast Saccharomyces cerevisiae' cited in the application see page 51 - page 69	2,6-8

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1993	17 -12- 1993

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	LE CORNEC, N

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEIN ENGINEERING. vol. 3, no. 2 , 1989 , EYNSHAM, OXFORD, ENGLAND GB pages 145 - 151 I.D. PETERS ET AL 'Biosynthesis of winter flounder antifreeze proprotein in E. coli' cited in the application see abstract	1,8
A	BIOCHIMICA ET BIOPHYSICA ACTA vol. 1089 , 1991 , ELSEVIER , AMSTERDAM. pages 345 - 351 A.C.A.P.A. BEKKERS ET AL 'The use of genetic engineering to obtain efficient production of porcine pancreatic phospholipase A2 by Saccharomyces cerevisiae' see the whole article	1-7
A	GENE. vol. 112 , 1992 , AMSTERDAM NL pages 163 - 170 P.L. DAVIES 'Conservation of antifreeze protein-encoding genes in tandem repeats' see the whole document	9
A	GENE. vol. 110, no. 1 , February 1992 , AMSTERDAM NL pages 25 - 31 T. ACHSTETTER ET AL 'A new signal peptide useful for secretion of heterologous proteins from yeast and its application for synthesis of hirudin' cited in the application see table I	1,3,4
Y	EP,A,0 255 153 (UNILEVER NV) 3 February 1988 cited in the application see the whole document especially examples 2 and 3	2,6-8
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 81 , 1984 , WASHINGTON US pages 5330 - 5334 G. A. BITTER ET AL 'Secretion of foreign proteins from Saccharomyces cerevisiae directed by alpha-factor gene fusions' see the whole document	1,3,4

1

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 12 , 25 April 1991 , BALTIMORE US pages 7339 - 7344 G. M. MUELLER ET AL 'Inhibition of recrystallization in ice by chimeric proteins containing Antifreeze domains' cited in the application see the whole document ---	1
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 259, no. 23 , 10 December 1984 , BALTIMORE US pages 14960 - 14965 B. GOURLIE ET AL 'Winter flounder Antifreeze proteins : A multigene family' see the whole document -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/EP 93/01969

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9013571	15-11-90	US-A-	5118792	02-06-92
		AU-A-	5814390	29-11-90
		EP-A-	0472662	04-03-92
		JP-T-	4505329	17-09-92
EP-A-0255153	03-02-88	AU-B-	602703	25-10-90
		AU-A-	7513287	11-01-88
		WO-A-	8707641	17-12-87
		US-A-	5082778	21-01-92
		JP-T-	1500242	02-02-89